IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Evans et al.

Appln. No.	09/763,076	Group Art Unit:	1638
Filed:	14th May 2001	Examiner:	M.A. Ibrahim

For: GENETIC METHOD FOR THE EXPRESSION OF POLYPROTEINS IN PLANTS

DECLARATION OF JASON VINCENT UNDER 37 C.F.R. §1.132

I, **Jason Vincent**, hereby declare that:

1. I am a citizen of the United Kingdom residing at 11 Hebbecastle Down, Warfield, Berkshire, RG42 2QD.

2. I am a graduate of University of East Anglia with a BSc degree in Molecular Biology and Genetics which was granted in 1994. I also received a PhD degree in Molecular Biology and Genetics from University of East Anglia in 2000. Since January 2000 I have been employed at the Jealott's Hill Research Station of SYNGENTA LTD, which is located in Bracknell, Berkshire in the United Kingdom. I am currently a team leader in the Trait Research Department and direct and conduct research into insecticidal protein toxins and their expression in plants

3. I am familiar with the subject-matter in the above-identified patent application and have reviewed its contents. I have also reviewed the Examiner's comments in the Office Action mailed 10th April 2003, in particular as they relate to the rejection of claims 1-12, 14-16 and 18-21 under 35 USC § 112 as being not enabled by the specification.

4. On page 7 of the Office Action the Examiner has cast doubts on the ability of a linker propeptide from a plant antimicrobial protein to mediate the expression of multiple proteins in plants when said proteins are anything other than antimicrobial proteins. I can confirm that under my direct supervision or control, the following experiments were carried out to determine the ability of the fourth internal propeptide of the IbAMP precursor to mediate the expression of a small insecticidal protein and β-glucoronidase as a cleavable fusion protein in plants.

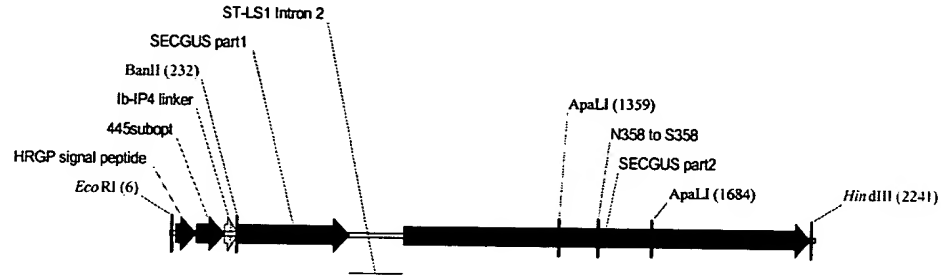
5. **Overview**: The experiments described herein were devised to demonstrate use of the *Impatiens balsamina* AMP polyprotein linker IP4 for expression of two distinct protein units, unrelated to antimicrobial peptides, from the same plant-expressed RNA message. The proteins used were the insecticidal peptide

known as "445" (see International Patent Publication No. WO 01/00841and the β-glucuronidase, or "GUS", protein.

6. **Construct Assembly**: The 445::IP4::secGUS fusion contructs were made in two parts. Firstly, the HRGP signal peptide coding sequence *(Z. mays* hydroxyproline-rich glycoprotein; Stiefel *et al.*, 1990, Plant Cell 2: 785-793). and 445 coding sequence (codon optimised, or, containing the *Solanum tuberosum* LS1a intron 2; Eckes *et al.*, 1986 Mol. Gen. Genet. 205:14-22) together with the IP4 coding sequence were created by recursive PCR using long, overlapping, oligonucleotide primers corresponding to the desired sequence of each part of the fusion. Full-length products were cloned into pCRbluntTOPO (Invitrogen) and the inserts sequenced entirely. Secondly, the secretable mutant of β-glucuronidase ("secGUS"; Farell &Beachy, 1990, Plant Mol. Biol. 15: 821-825) was created by PCR site-directed mutagenesis using two sets of primer pairs where one primer was mutagenic and the other non-mutagenic, resulting in two products corresponding to the first or second half of the uidA coding sequence. An intronic uidA sequence was used as a template. The two sub-fragments were spliced together using PCR overlap extension firstly by denaturing an equimolar mixture of the two at 94^0C. Then in the absence of additional primers, but in the presence of dNTPs, Pfu DNA polymerase and the appropriate buffer, the mixture was cooled to an annealing temperature of 52^0C, before raising the temperature to 72^0C to effect polymerase extension. This temperature cycle was repeated three times. Following this step, an aliquot of this mixture was used as template in a conventional PCR reaction where flanking primers were included to allow amplification of a full-length, mutated, uidA product. This product was cloned into pCRbluntTOPO and sequenced entirely. A clone containing the desired mutation was selected for further use.

Plasmid pCRbluntTOPO containing the two 445::IP4::secGUS fusions were digested with BanII and EcoRI restriction endonucleases and the liberated insert fragments were cloned into pUC18 (pre-digested with the same enzymes). This created pUC18 acceptor plasmids containing the 445::IP4::secGUS fusions. Subsequent digestion of these acceptor plasmids with BanII and KpnI was followed by ligation with a BanII/KpnI fragment prepared from the pCRbluntTOPO::secGUS clone described above. The resulting constructs contained the fusions as depicted in figures 1 and 2.

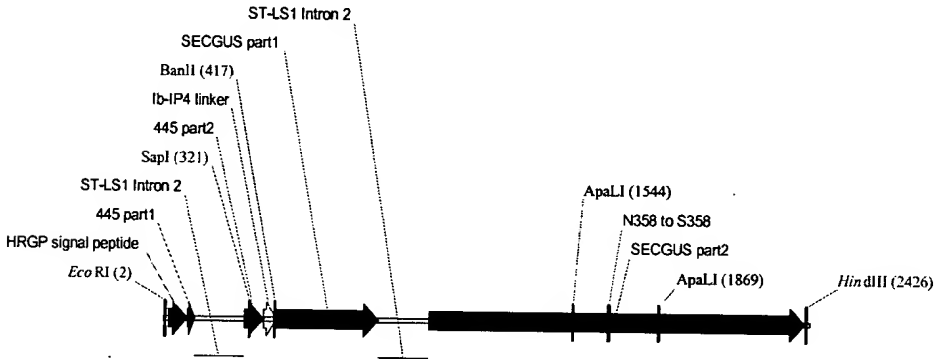
Figure 1.



HRGP445subopt +IP4+SECGUS

2255 bp

Figure 2.



445LS1aIP4+SECGUS

2440 bp

The unique SpeI and HindIII flanking sites in these two pUC constructs were used to liberate the fusion sequences and transfer them into the plant transformation vector p24-1 (Figure 3).

E.coli cells were transformed with the final plant transformation constructs (DNAs) and cultures of transformed *E. coli* were grown, harvested and the plant transformation constructs were isolated using the Qiagen MaxiPrep method. Introduction of these DNAs into maize callus or protoplast cell lines was performed as described by Keappler *et al* (1992, Theor. Appl. Genet. 84:560-566), and Shukhapinda *et al* (1993, Plant Cell Reports 13:63-68). A positive control *uidA* transformation construct (non-secretable GUS) was also used for comparative GUS fluorometric experiments (Figure 4).

Figure 3.

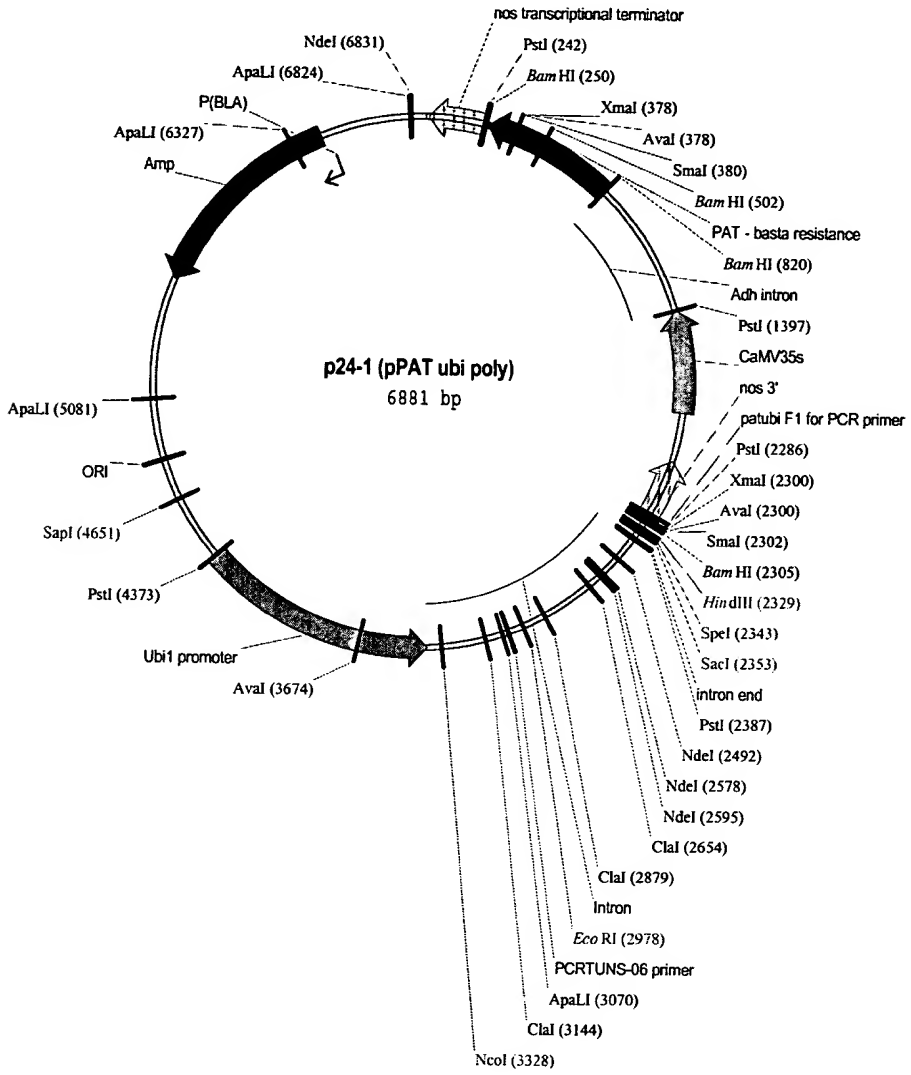
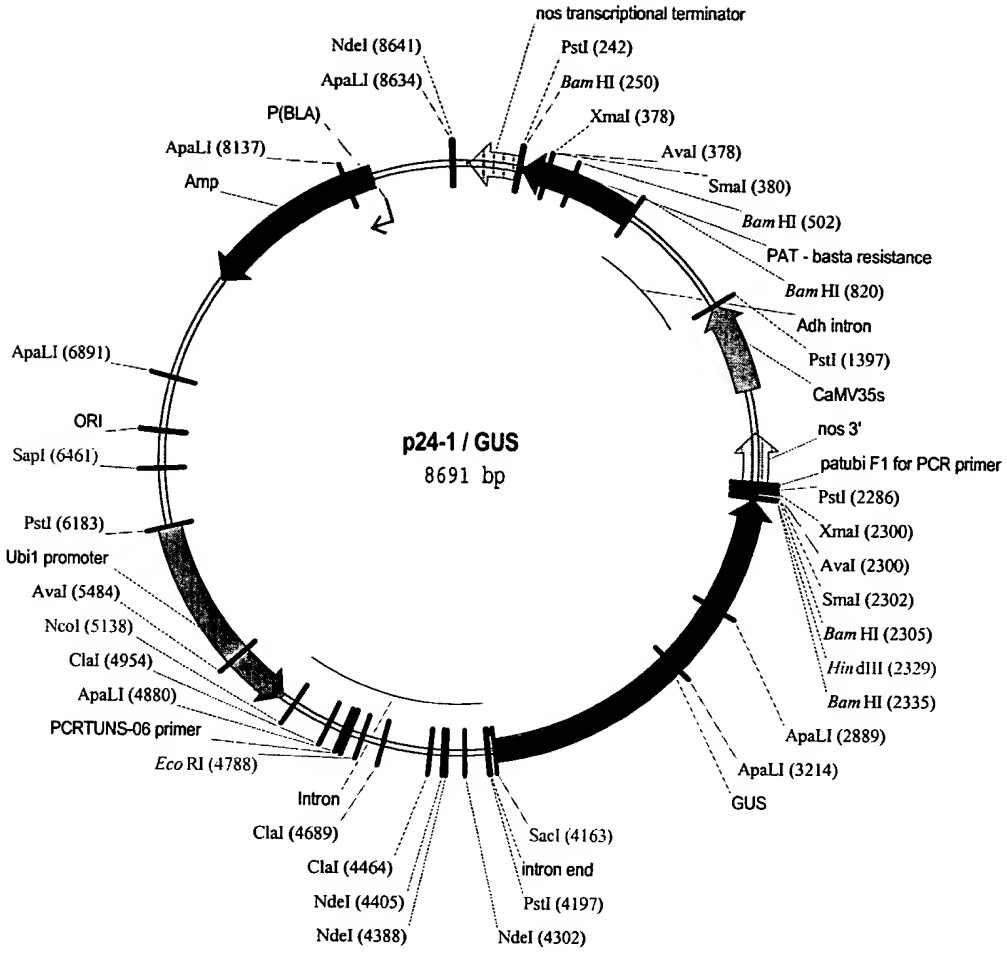

p24-1 (pPAT ubi poly)
6881 bp
nos transcriptional terminator
NdeI (6831)
PstI (242)
ApaLI (6824)
BamHI (250)
P(BLA)
XmaI (378)
ApaLI (6327)
AvaI (378)
Amp
SmaI (380)
BamHI (502)
PAT - basta resistance
BamHI (820)
Adh intron
PstI (1397)
CaMV35s
nos 3'
patubi F1 for PCR primer
PstI (2286)
XmaI (2300)
AvaI (2300)
SmaI (2302)
BamHI (2305)
HindIII (2329)
SpeI (2343)
SacI (2353)
intron end
PstI (2387)
NdeI (2492)
NdeI (2578)
NdeI (2595)
ClaI (2654)
ClaI (2879)
Intron
EcoRI (2978)
PCRTUNS-06 primer
ApaLI (3070)
ClaI (3144)
NcoI (3328)
ApaLI (5081)
ORI
SapI (4651)
PstI (4373)
Ubi1 promoter
AvaI (3674)

Figure 4.



7. **Results – β-glucorinidase activity** GUS activity in transformed maize samples was measured using the increase in fluorescence at 455nm as a result of conversion of the fluorogenic GUS substrate 4-methyl umbelliferone (4-MU) (Jefferson, 1987 EMBO J. 13, 3901–3907). Table 1 shows data for maize protoplasts expressing the fusion constructs. The data suggests that there is scorable GUS activity in these examples, inferring that the fusion constructs are capable of directing recombinant, functional, GUS expression in transformed maize cells.

Table 1.

Reporter Gene Construct		nMolMU/h*mg
experiment 1		
p24-/GUS	Pellet	11154.5
p24-/GUS	Supernatant	36302.8
p24-/HRGP-445 IP Sec GUS	Pellet	2474.3
p24-/HRGP-445 IP Sec GUS	Supernatant	21700.6

experiment 2		
p24-1 / HRGP - 445 suboptimised - IP4 - secGUS	Pellet	189.3
p24-1 / HRGP - 445 suboptimised - IP4 - secGUS	Supernatant	247.2
p24-1 / HRGP - 445 + LS1a - IP4 - secGUS	Pellet	129.9
p24-1 / HRGP - 445 + LS1a - IP4 - secGUS	Supernatant	112.6
p24-1 / GUS	Pellet	586.7
p24-1 / GUS	Supernatant	582.0

8. **Results - SELDI TOF mass spectrometry**

SELDI TOF MS was used to determine the type of 445 peptides that were produced in extracts of maize cells transformed with the fusion constructs. 445-specific antibody was linked to the surface of a Protein chip (Ciphergen Systems) and native protein extract from transformed cells was applied to the chip surface. After washing the chip to remove unbound proteins, the chip was analysed in a Ciphergen SELDI unit.

Data for line 36A (callus) which had been transformed with the HRGP-445LS1aIP4secGUS construct indicated that a mixture of 445 peptide species were present, containing variable amounts of the IP4 linker. 445-specific peaks were as follows:-

3381Da = 445 + serine or 445 minus N-term glycine+lysine plus serine+asparagine+alanine
3495Da = 445 + serine+asparagine
3566Da = 445 + serine+asparagine+alanine
3637Da = 445 + serine+asparagine+alanine+alanine

Data for line 108A (callus) which had been transformed with the HRGP-445suboptIP4secGUS construct indicated that a single 445 species was present:-
3437Da = 445 minus N-term glycine plus serine+asparagine.

These data indicate that the fusion constructs are capable of directing expression of the 445 protein unit in transformed maize cells, and that the 445 protein units must have been produced by cleavage of a 445::IP4::secGUS protein precursor by cellular proteases.

9. **Results - Western blot data**

Western blotting (immuno-blotting) of protein extracts from fusion construct-expressing maize callus lines using a 445-specific antibody demonstrated that in lines 84A, B and 85A (HRGP-445suboptIP4secGUS, and HRGP-445LS1aIP4secGUS constructs, respectively) cross-reacting bands of the correct size for 445 were detected. This observation further supports the observations made in SELDI TOF experiments above.

10. The experiments and results described herein demonstrate that the IP4 linker can be employed to achieve expression of proteins other than those of

antimicrobial function in transgenic plant cells. Furthermore, the data presented demonstrates, by two independent methods, that the full-length fusion protein is cleaved at the IP4 linker to release the component protein units.

11. All statements made herein of my own knowledge are true, and all statements made on information and belief are believed to be true. Further, these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Here ends my declaration

Jason Vincent Date